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Thermo-sonic assisted enzymatic pre-treatment of sludge biomass as potential feedstock for oleaginous yeast cultivation to produce biodiesel

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ABSTRACT

Solubilization of activated sludge is a crucial process before its use as an appropriate renewable feedstock for biofuel generation which could be a legitimate alternative arrangement for contemporary concerns on fuel crisis, climate change and food security. The present study investigates the thermo-sonic assisted enzymatic pre-digestion of municipal waste activated sludge (MWAS) to cultivate oleaginous yeast *Naganishia liquefaciens* NITTS2 to produce lipids for biodiesel production. The maximum suspended solids reduction and sCOD observed were 36.42 ± 0.7 and 41.35 ± 0.5%, respectively at optimum conditions. The pre-digested sludge was used as a nutritional medium for yeast cultivation and the obtained maximum biomass and lipid content were 17.85 ± 0.64 g/L and 65.43 ± 1.60%, respectively. The consumption of nutrients present in the medium was analyzed before and after the batch cultivation. Lipid extraction was optimized using ultrasonication at different temperature and its characteristic profile was analyzed by GC-MS. Fatty Acid Methyl Esters (FAMEs) was produced (88.45 ± 1.2%) through enzymatic transesterification and further confirmed by ^{1}H NMR spectroscopy. Thus, the combined pre-digestion would help to improve the solids reduction in the MWAS and the solubilized sludge could be used as a renewable substrate for biodiesel production.

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1. Introduction

Wastewater treatment plants are increasing in number all over the world due to increase in population, environmental awareness and need for good water resources. Various physicochemical and biological methods [1] are adopted for the municipal wastewater treatment. Waste activated sludge is the primary by-product from biological methods and since large quantities of sludge are generated, handling of the sludge consumes nearly 60% of the total plant operating cost [2]. Although anaerobic digestion is commonly used to stabilize the sludge and reduce the volume, this process seems to be ineffective due to its requirement of large reactors and its sluggishness. Hence, sludge management is a strenuous task, but with a novel approach, the sludge could be an economically beneficial resource for the production of value-added products.

Rapid growth in technological advancements and industrialization leads to consumption of phenomenal quantities of energy. Various renewable fuels are being developed to meet this demand, and also to prevent deleterious effects on the environment. Among those, biodiesel derived from renewable biomass resources is a potential alternative because of less toxic nature, ability to run existing engines and blending properties [3]. It produces energy density close to petroleum fuels and has appreciable lubricating characteristics [4]. Many countries are modifying their regulations to use a biodiesel blend [5]. The use of edible products like palm, rapeseed, sunflower, soybean, coconut oil and canola as lipid feedstock increases the cost of biodiesel production [6–8]. Instead, microbial oils, which could be produced with less labor, easier scale up and shorter life cycle, can be utilized as cost-effective feedstock. This could also address the issues of agricultural land and food supply demands [9].

The microbial oil or the single cell oil (SCO) is obtained from lipid storing organisms (oleaginous) such as yeast, microalgae, bacteria or mold. Especially, yeast attracts more interest due to the high growth rate, increased cell density and ability to store lipids up to nearly 70% of their dry weight [10]. These lipids from the yeast can also be used as an edible oil source [11] and for the production

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of oleochemicals and additives used in cosmetic or food industries. Other than lipids, they produce nutrients and value-added products including proteins and carbohydrates [12]. One of the major factors impacting the industrial scale culturing of these yeasts is the availability of low-cost nutrient medium or substrate.

The waste activated sludge generated by municipal wastewater treatment plants (MWTPs) is composed of various nutrients including proteins, carbohydrates, fibres and other micro-nutrients [13]. Therefore, it could be a sustainable and cheap feedstock for microbial culture to produce lipids after appropriate pre-treatment. Some oleaginous yeasts are found to be capable of effectively consuming these resources as a substrate due to the synthesis of hydrolytic enzymes [14]. Pre-treatment methods including mechanical, chemical, thermal and their combinations facilitate the breakdown of complex organic compounds in municipal waste activated sludge (MWAS) into simple compounds, which can be easily consumed by the cultured lipid producing organisms [15–18].

The ultrasonic pre-treatment of sludge, one of the mechanical methods, was found to be a very effective method and was evaluated in detail using the laboratory to large-scale reactors [19,20]. The ultrasonic waves, which are above human hearing range, produce repeated compressions and rarefactions when immersed in the liquid [21]. This results in regions with high negative pressure and cavitation (microbubbles generation). When these bubble break, they produce an immense quantity of localized temperature (up to 5000 K) and high pressures (up to 180 MPa) exerting hydromechanical shear forces [22,23], which are responsible for the sludge disintegration and release of intracellular components into the liquid phase. The quantity of dissolved organic compounds is significantly increased due to sludge degradation [24,25] and further, the efficiency of pre-treatment could be enhanced by combining with thermal treatment [20], alkalization or enzymatic treatment. Such enzymatic treatment could be carried out by using commercial enzymes or multi-hydrolytic enzyme from low-cost sources (garbage enzymes). These garbage enzymes can perform a combined hydrolytic activity with good efficiency and it is an eco-friendly process.

From the above literature survey, it is concluded that no attempt has been made for thermo-sonic assisted enzymatic pre-digestion of MWAS till date and therefore, the utilization of such pre-digested MWAS as a nutrient medium for the synthesis of lipids using oleaginous yeast for biodiesel production is a novel idea. The main objectives of the present study are to (i) investigate and optimize the impact of temperature on MWAS solubilization; (ii) optimize the ultrasonic power input and time for effective sludge disintegration; (iii) study the influence of thermo-sonic assisted enzymatic hydrolysis of sludge on solids reduction and organics release; (iv) explore the suitability of pre-digested sludge for yeast cultivation by batch process; (v) optimize the lipid extraction using ultrasonication and characterize the obtained lipids by GC-MS; (vi) produce biodiesel from the yeast lipids using enzyme catalyst.

2. Materials and methods

2.1. Materials

The Yeast Peptone Dextrose (YPD) agar and broth purchased from Himedia chemicals, Mumbai, India were used to maintain yeast culture. The microbial lipid was extracted and transesterified using the solvents, chloroform (>99%) and methanol (>99%) purchased from Sigma Aldrich, Chennai, India and analytical grade 95–97% sulphuric acid (H_2SO_4) purchased from Merck Specialities Pvt. Ltd., Mumbai, India. All other reagents and chemicals used were of analytical grade and purchased from Sigma Aldrich or Merck, India.

2.2. Thermo-sonic pre-digestion

MWAS was collected in the secondary clarifier after partial thickening by flotation from the MWTP in Madurai, Tamil Nadu, India. The collected sample was immediately taken to the laboratory and kept at 4 °C until further use. 200 mL of sludge was taken in a 250 mL doubled walled beaker with water circulation to maintain the required temperature. The sample was exposed to ultrasonication (20 kHz frequency) using probe-type sonicator (Lark Classic Model, Lark innovative Fine Teknowledge Ltd., Chennai, India) with different sonication power input ranging from 10 to 100 W for different time intervals viz. 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 min at different temperature. The control sample was not subjected to ultrasonication. The effect of pre-digestion conditions of thermo-sonic process on sludge solubilization was optimized based on these above experimental parameters. The assessment of the efficiency of MWAS digestion was performed by COD solubilization (Eq. (1)) and suspended solids (SS) reduction (Eq. (2)).

$$\text{COD solubilization}(\%) = \left(\frac{\text{sCOD}_a - \text{sCOD}_i}{\text{TCOD}_i - \text{sCOD}_i}\right) \times 100 \quad (1)$$

$$\text{SS reduction}(\%) = \left(\frac{\text{Before treatment SS} - \text{After treatment SS}}{\text{Before treatment SS}}\right) \times 100 \quad (2)$$

where $sCOD_a$ is the soluble chemical oxygen demand (sCOD) of the sludge after pre-digestion (mg/L), $sCOD_i$ is the sCOD concentration of the sludge before pre-digestion (mg/L) and $TCOD_i$ is the total chemical oxygen demand (TCOD) of the sludge before pre-digestion (mg/L).

The energy supplied (ultrasound power) per unit mass of biomass was calculated as

$$E_{SE}(\text{kJ/kg}) = \frac{P \cdot t}{V \cdot TS} \quad (3)$$

where E_{SE} is specific energy input in (kJ/kg TS), P is power required for ultrasonication (kW), t is the time needed for effective disintegration (s), V is the volume of treated sludge (L), TS is the initial total solid concentration (mg L^{-1}).

2.3. Thermo-sonic assisted enzymatic hydrolysis

After thermo-sonic digestion at the optimized conditions, the sludge sample was further exposed to the enzymatic treatment. The production and characterization of multi-hydrolytic garbage enzyme (MGE) were reported in our earlier study [26]. For this study, the MGE was produced through anaerobic fermentation using 15 min ultrasonic pre-treated organic solid waste (Orange, Sweet Lime, Pineapple and Pomegranate fruit peels) at optimal conditions of pH 6.2, 37 °C, 216 rpm and 4.9 days. To assess the effect of enzyme dosage on MWAS hydrolysis, 5–50% v/v of MGE was added to 50 mL of thermo-sonic pre-digested MWAS in a beaker and exposed to a different temperature (30–50 °C) under constant stirring speed (200 rpm) up to 40 h. The samples were taken and analyzed at a regular time interval of every 4 h. The hydrolytic conditions of enzymatic process on sludge solubilization were optimized based on these experiments.

2.4. Yeast strain, culture conditions and inoculum preparation

The yeast strain *Naganishia liquefaciens* NITTS2, isolated and identified in our earlier study [27], was used in this study. It was sustained on YPD agar slants at 4 °C and it was sub-cultured twice in a month [28]. A seed culture was prepared by separately inoculating one loop of the isolate in 10 mL of YPD broth medium (growth medium) and then incubating at 27 °C under stirring of 100 rpm for 48 h in the incubator. The final cell concentration in the seed culture was adjusted to 10^6 cells/mL using sterilized deionized water. The inoculum was prepared by transferring 1 mL of seed culture into 50 mL YPD broth medium in a 100 mL Erlenmeyer flask and incubating at 27 ± 2 °C in the orbital shaker at 130 rpm for 48 h. All these growth media were sterilized at 121 °C for 15 min at 15 psi before use [29].

2.5. Growth dynamics, biomass and lipid productivity

The yeast strain was cultivated in thermo-sonic assisted enzymatic pre-digested (TSEP) sludge medium. 200 mL of filtered medium was taken in 250 mL Erlenmeyer flask and autoclaved. Then, 10% (v/v) of seed culture was added in the flask and was incubated at 30 ± 2 °C using an orbital shaker incubator with pH 6.3 ± 0.2 under constant stirring (160 rpm) for 168 h. These culture conditions were optimized for this strain in our previous study [27]. The specific growth rate was determined by measuring Optical Density (OD) at 600 nm using spectrophotometer (UV–Vis, Shimadzu UV-1601, Mumbai, India) at regular time intervals and the growth dynamics were estimated from the measured values. The specific growth rate (μ) [30] was calculated as

$$\mu = \ln\left(\frac{OD_{t2}/OD_{t1}}{t2 - t1}\right) \quad (4)$$

where OD_{t1} and OD_{t2} are the optical densities at the beginning (t1) and the end (t2) of the logarithmic growth phase, respectively.

To determine the biomass productivity (Pdwt), 3 mL of sample was taken from cultivation flask and yeast cells were separated by centrifugation for 10 min at 8000 rpm with 4 °C using a cooling centrifuge (MPW-352/R/RH Centrifuge, MPW MED Instruments, Poland). Then, it was freeze-dried using lyophilizer (4 kg model, Lark Innovative Fine Teknowledge, Chennai, India) till constant weight was attained and the dry biomass weight was gravimetrically determined. The lipid was extracted from the biomass [31]. The lipid yield (Ly) and volumetric lipid productivity (VLp) were calculated as follows:

$$\text{Ly (dwt\%)} = \left(\frac{\text{Lipid obtained (g)}}{\text{Biomass used (g)}}\right) \text{x } 100 \quad (5)$$

$$\text{VLp} = \text{Pdwt x Ly}/\ 100 \quad (6)$$

2.6. Ultrasonic-assisted lipid extraction and analysis

5 g of dry cell biomass was suspended in 100 mL of extracting solvent (1:1 ratio of chloroform and methanol) in a 250 mL doubled walled beaker with water circulation to maintain the required temperature. The sample was exposed to ultrasonication for lipid extraction using a probe-type sonicator (Model: Lark Classic Model, probe diameter: 6 mm, frequency: 20 kHz, power: 45%). The effect of temperature (20–40 °C) and sonication time (5–30 min) on lipid extraction were investigated. The control sample was not subjected to ultrasonication. Subsequently, the sample was centrifuged at 8000 rpm for 10 min, and the supernatant was filtered through a 0.45 μm syringe filter. Profile of lipid was analyzed by GC-MS (Perkin Elmer Clarus 500, Turbomass version 5.2.0) after evaporating the solvent in the sample using a rotary evaporator. Fatty acids were separated by Capillary Column Elite-5 (column length 30 m; inner diameter 250 μm). The column was equipped with a Scion SQ GC-MS Single Quadrupole mass spectrometer (mass detection range 40–450 amu). A programmed temperature vaporizer (PTV) injector system was used for fixing the mass transfer line (200 °C) and ion source temperatures (160 °C). The CP-8410 auto-sampler system was used with helium as the carrier gas (ultra-high purity, 99.99%) at a constant flow rate of 1 mL/min. The injector temperature maintained was 280 °C, and the column temperature was initially kept at 60 °C, then gradually increased to 150 °C at a rate of 6 °C/min, held for 2 min and finally raised to 280 °C at a rate of 4 °C/min, held for 5 min. The sample (dissolved in hexane) of 1 μL was injected into a column using a split mode with a split ratio of 1: 10. An electron ionization system with ionization energy of 70 eV was used for the detection purpose and the mass spectra generated was matched with those of standards available in the mass spectrum library (NIST 2005) [31].

2.7. FAMEs production and analysis

The enzymatic transesterification was performed by following the method described by Selvakumar and Sivashanmugam [27] to produce Fatty Acid Methyl Esters (FAMEs), i.e., biodiesel using garbage lipase as a biocatalyst. The production and characterization of the garbage lipase have been elucidated in our earlier work [32]. The solvent in the produced FAMEs was removed using a rotary evaporator and then the sample was subjected to hot water washing three times to remove impurities [33]. The resultant biodiesel from the yeast lipids was characterized by using Bruker AVANCE III 500 MHz (AV 500) multi-nuclei solution ^{1}H NMR spectrometer. Deuterated chloroform ($CDCL_3$) and tetramethylsilane (TMS) were used as a solvent and internal reference, respectively. The system consisted of 5 mm broadband gradient probe with variable temperature range from −150 to +180 °C. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 298 K, with a pulse angle of 30°, recycle delay of 1 s for a specified number of scans. The purity of FAMEs was calculated according to the ^{1}H NMR spectrometer values [32] using Eq. (7).

$$C = \frac{2A_{ME}}{3A_{CH2}} \times 100 \quad (7)$$

where C is the percentage of methyl ester in the sample, A_{ME} and A_{CH2} are the integration value of the methoxy protons and α-methylene protons of the methyl esters, respectively.

2.8. Analytical methods and statistical tools

The soluble proteins and carbohydrates were analyzed in MWAS sample by Lowry and phenol-sulphuric acid method, respectively [34]. The concentration of micronutrients present in the TSEP medium was estimated with the help of water and wastewater analysis kit (Palintest Water Analysis Technologies, UK) using Photometer 7100 model. All the assays were conducted in triplicate and the results were taken an average of three values. The standard deviation is represented in the error bar. One-way ANOVA analysis followed by standard *t*-test was applied to test the significance of results and $p < 0.05$ was measured to be statistically significant.

3. Results and discussion

3.1. Thermo-sonic pre-digestion of MWAS

Pre-digestion of MWAS is necessary to make it as a utilizable nutrient for microbial cultivation. The physicochemical characterization of the raw MWAS used in this study was analyzed and reported in our earlier work with the results of pH, 6.4; Initial Total Solids (TS), 11600 ± 300 mg/L; Suspended Solids (SS), 7000 ± 300 mg/L; Volatile Suspended Solids (VSS), 5600 ± 200 mg/L; Total Dissolved Solids (TDS), 4800 ± 200 mg/L; Total COD (TCOD), 10,600 ± 300 mg/L; Soluble COD (sCOD), 200 ± 70 mg/L; sProteins (mg/g dwt), 41.7 ± 3.5; sCarbohydrates (mg/g dwt), 25.2 ± 2.8. The Elemental composition (%) of the total solids was found to be C (35.3 ± 0.2), H (5.8 ± 0.1), N (6.8 ± 0.1) and S (0.1 ± 0.1) [18]. The thermo-sonic pre-digestion of MWAS was performed with different sonication power input at various temperature for 45 min. The efficiency of the proposed method was assessed based on the increment in the sCOD percentage of the sample.

3.1.1. Effect of sonication power input on COD solubilization

Ultrasonic pre-digestion is an effective methodology for the disintegration of biomass [19,35]. The optimization of power utilization for sludge disintegration is essential for the profitable commercial exploitation of the pre-digested MWAS [36]. The influence of different sonication power input on COD solubilization is presented in Fig. 1a.

It can be seen from this figure that the COD solubilization is gradually increased up to 80 W sonication power input and then less increment (<0.5%) is observed from 80 to 100 W. Similar behavior was reported for the disintegration of secondary sludge using different sonication power input [37]. The increase in sonication power input creates stronger hydro-mechanical shear forces causing enhancement of sludge digestion. At low ultrasonic frequencies (20 kHz), the hydro-mechanical shear forces are strong enough for the fragment of sludge flocs, breakdown of cell walls and membranes resulting in a reduction in biodegradability time as well as an increase in biofuel production. Hence, the intracellular substances can be released into the surrounding aqueous sludge medium [38,39]. The maximum COD solubilization at 80 W sonication power input achieved was 4.6 ± 0.5% at ambient temperature. To enhance the digestion efficiency, sonication would be performed with the combination of other pre-digestion techniques namely thermalization. Thus 80 W sonication power input was considered as optimum and used in further investigations.

3.1.2. Effect of temperature on COD solubilization

As the temperature is a crucial parameter for sludge stabilization and ousted of soluble organic fractions in the liquid phase during pre-treatment [40], the influence of temperature on thermo-sonic pre-digestion was investigated at different temperature ranging from 30 to 90 °C. Fig. 1a depicts the impact of temperature on sludge disintegration and it is observed that the COD solubilization is increased gradually with increase in temperature. The solubilization of organic fractions is increased by the breakage of chemical bonds of sludge cells due to thermal effect [41]. The maximum COD solubilization obtained for 30, 40, 50, 60, 70, 80 and 90 °C respectively were 4.6 ± 0.5, 7.3 ± 0.5, 8.5 ± 0.7, 12.3 ± 0.7, 15.6 ± 0.6, 20.6 ± 0.8 and 21.5 ± 0.7%. The increased SCOD trend with an increase in temperature is consistent with the outcome of Serkan et al. [37]. As seen from Fig. 1a that the COD solubilization is enhanced with an increase in temperature up to 80 °C and a further rise in temperature to 90 °C has no significant improvement in sCOD. It was statistically proven by one-way ANOVA technique followed by a standard *t*-test at $p < 0.05$ level. It revealed that there was no significant difference in the sCOD release (p-value = 0.7365, see Table A1 in supplementary data) between the temperature of 80 and 90 °C. The calculated p-value for COD solubilization between 70 and 80 °C was 0.2817 (see Table A2 in supplementary data) and it indicates that the measured sCOD values were not closer together. It reveals that release of both intra- and extracellular substances and their stabilization were largely improved at the temperature of 80 °C. The COD solubilization (20.6 ± 0.8) with the assistance of thermal effect (80 °C) is 4.48 times greater than ultrasonic disintegration (4.6 ± 0.5) alone. This may be due to the synergistic effect of combined two different sludge pre-digestion mechanisms. Based on the chemical parameters and statistical results, the optimum temperature for effective sludge digestion in thermo-sonication process was concluded as 80 °C and this was used for subsequent studies.

3.1.3. Influence of pre-digestion time on SS reduction and sCOD

In the pre-digestion process, the time taken for accomplishing a highest SS reduction and sCOD release are the indispensable parameter in the economic aspect. The effect of treatment time on SS reduction and COD solubilization is illustrated in Fig. 1b.

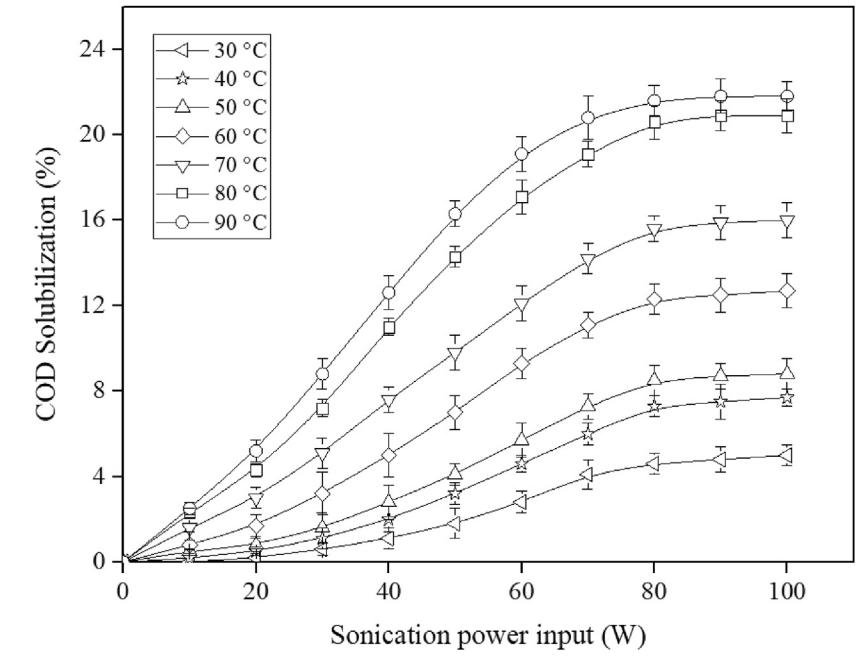
It can be seen from this figure that there is a reduction in SS concentration from 7000 to 6030 mg/L with the increase in a treatment time of 0–30 min. From the treatment time, 30–50 min no considerable increment in SS reduction is observed. Similarly, the COD solubilization is increased up to 30 min of pre-digestion time and negligible improvement is detected beyond this time. The determination of energy utilized per unit mass of sludge is important for the large-scale exploitation of the disintegrated MWAS [36]. The highest sCOD release and SS reduction percentage determined respectively were 20.60 ± 0.8 and 13.86 ± 0.7 with the specific energy input of 6000 kJ/kg at a treatment time of 30 min. In this study, the improved percentage of COD solubilization and SS reduction were achieved with minimal specific energy when compared to other pooled pre-digestion methods (Alkaline + Sonication) [42]. Thus, for the effective disintegration of sludge 80 W, 80 °C and 30 min treatment time was considered as an optimum condition in the thermo-sonic pre-digestion process.

3.2. Thermo-sonic followed by enzymatic hydrolysis of MWAS

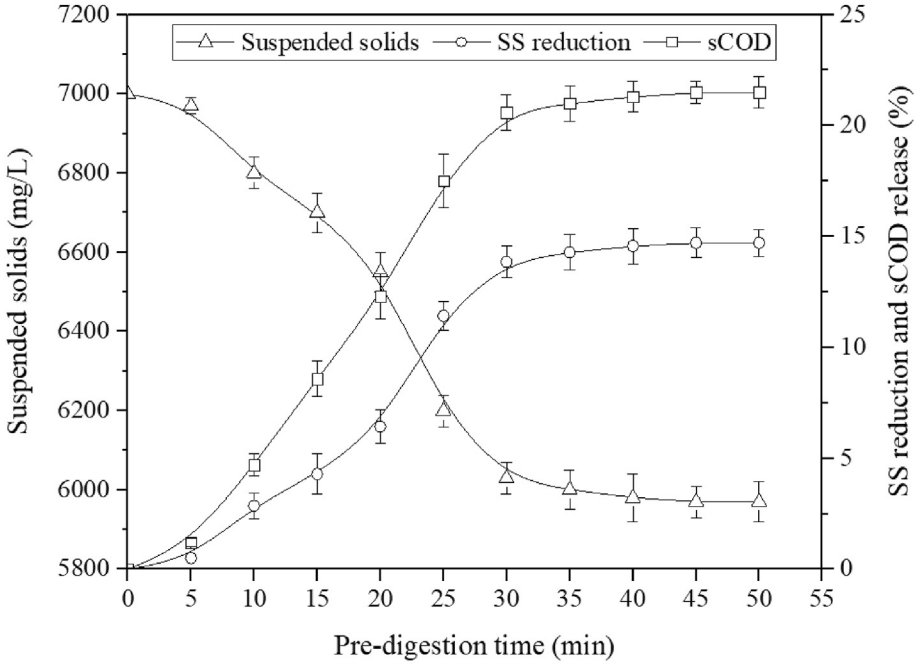
3.2.1. Effect of temperature on sludge pre-digestion using MGE

Sample with the optimized condition of thermo-sonic digestion was further exposed to the enzymatic treatment. As the temperature is an important parameter for the liberation of soluble organics in the liquid phase and stabilization of sludge during the pre-digestion [40], the influence of temperature on SS reduction and COD solubilization was performed at a different temperature ranging from 30 to 50 °C. Fig. 2 displays the effect of temperature on sludge disintegration, and it can be seen from this figure that the concentration of solids reduction and sCOD release are increased gradually with increase in temperature from 30 to 40 °C, and is decreased at 50 °C.

The reason for decreased solubilization at 50 °C may be due to partial denaturation of the hydrolytic enzyme. From 30 to 40 °C, no significant difference in both solids reduction and sCOD release were observed and this was proven statistically also using one-way ANOVA technique followed by a standard *t*-test at $p < 0.05$ level. The calculated p-value was 0.3180 (see Table A.3 in supplementary data) for SS reduction and 0.4224 (see Table A.4 in supplementary data) for COD solubilization. Whereas from 40 to 50 °C, a significant difference was achieved in SS reduction (p-value = 0.0467, see Table A.5 in supplementary data) and sCOD release (p-value = 0.0438, see Table A.6 in supplementary data). The maximum sCOD were determined respectively to be 37.4 ± 0.4,



a



b

Fig. 1. a: Thermo-sonic pre-digestion of MWAS: Effect of **s**onication power input on sCOD release at different temperature. **b:** Thermo-sonic pre-digestion of MWAS: Influence of ultrasonication treatment time on SS reduction and sCOD release.

41.35 ± 0.5 and 31.00 ± 0.8%, and the solids content of 6030 ± 40 mg/L was reduced respectively to 4850 ± 40, 4450 ± 50 and 5250 ± 50 mg/L at 30, 40, and 50 °C. According to the experimental and statistical analysis, the multi-hydrolytic garbage enzyme can be applied for better hydrolysis of sludge sample in the temperature range of 30–40 °C. Thus, the optimum temperature for enhanced hydrolytic activity was concluded as 40 °C and this was used for further study investigations.

3.2.2. Effect of MGE dosage on MWAS solubilization

The MGE used for sludge hydrolysis was having the activity of protease (75.57 ± 1.82 U/mL), amylase (64.20 ± 1.04 U/mL), and lipase (52.05 ± 2.40 U/mL). To assess the efficiency of sludge fragmentation and bioavailability of soluble organic fractions in the

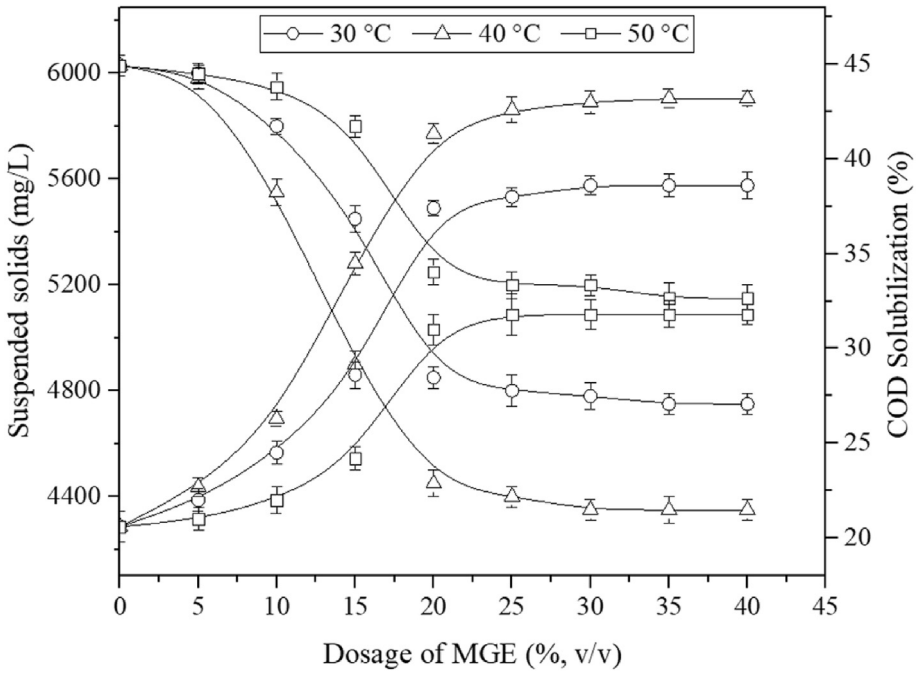


Fig. 2. Effect of MGE dosage on MWAS pre-digestion at different temperature (30, 40 and 50 °C).

sludge, the observation of change in SS reduction and sCOD release are considered to be important parameters [43]. The obtained results of pre-digestion of MWAS for different enzyme dosage (5–40%, v/v) is illustrated in Fig. 2. It can be observed from this figure that the solids concentration is decreased and the COD solubilization is increased with increasing of MGE dosage up to 20% and then the negligible improvement is noticed in both the cases. The similar trend was reported by Selvakumar and Sivashanmugam [26] in the hydrolysis process using un-treated MWAS. The non-availability of the appropriate substrates in the sludge sample for the enzyme may be the reason for the insignificant rise in solubilization when the MGE dosage used is >20%. The SS concentration of 6030 ± 40 mg/L was measured maximally reduced to 4450 ± 50 mg/L at 20% MGE dosage with the digestion temperature of 40 °C. The maximum achieved SS reduction and sCOD were found respectively to be 36.42 ± 0.7 and 41.35 ± 0.5%. The SS reduction and sCOD achieved in thermo-sono-chemical pre-digestion process were 20 and 27.6%, respectively [18]. Hence, this pre-digestion process exhibited better results due to combined multi-hydrolytic activities of the MGE.

3.2.3. *Influence of pre-digestion time on soluble organics release*

The MWAS typically consisted of macromolecules namely proteins and carbohydrates, which mainly contributes to the soluble COD of sludge [44]. Hence, the solubilization of proteins and carbohydrates were observed at different time intervals in pre-digestion of MWAS using MGE and is presented in Fig. 3a.

It is perceived that the fraction of organics increases as time increases up to 24 h and negligible improvement is observed beyond this time. The maximum soluble proteins and carbohydrates obtained respectively were 924 ± 10 and 667 ± 12 mg/L, at 24 h with 20% of MGE concentration. The amount of solubilized protein was 27.81% higher than carbohydrate released, which may be due to the fact that the existence of a higher proportion in the chosen activated sludge. Fig. 3b represents the change in solids reduction and COD solubilization with respect to pre-digestion time.

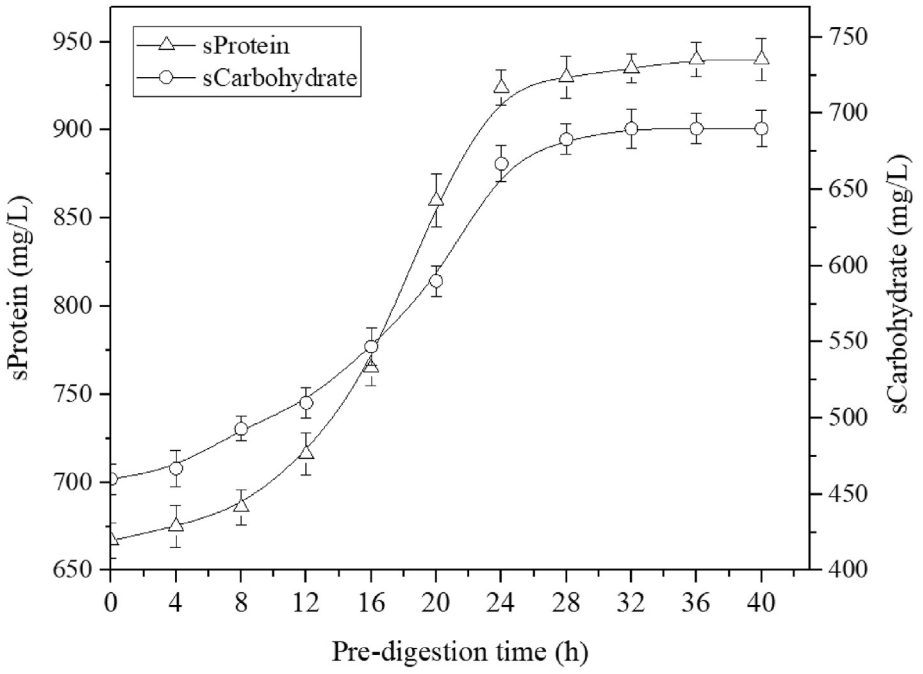
It is noted from Fig. 3b that the percentage of SS reduction and sCOD are increased significantly up to the treatment time of 24 h with the maximum achieved respectively were 36.42 ± 0.6 and 41.35 ± 0.5%. Beyond 24 h pre-digestion time, only an insignificant increment os observed in both the cases. Thus, the pre-digestion time required to obtain maximum solubilization of sludge at optimum conditions (20% v/v MGE dosage and 40 °C temperature) is concluded to be 24 h.

3.3. *Comparison of different pre-digestion methods on MWAS solubilization*

In this study, different sludge disintegration methods namely thermo-sonic pre-digestion (TSP) and TSP followed enzymatic pre-digestion (TSEP) were systematically evaluated based on the solids reduction, COD solubilization and fraction of organic release in the MWAS. The enzymatic pre-digestion (EP) and its influence on MWAS disintegration were reported in our previous work [26]. The obtained maximum percentage of SS reduction and sCOD from different pre-digestion methods are illustrated in Fig. 4a.

The solids concentration reduction was increased respectively by 13.86 ± 0.7, 28.9 ± 0.8 and 36.42 ± 0.6% for TSP, EP and TSEP. Similarly, the sCOD was increased respectively by 20.6 ± 0.8, 32.0 ± 1.3 and 41.35 ± 0.5% for TSP, EP and TSEP. The solids reduction measured in the TSEP process was 22.56% higher than TSP and 7.52% higher than EP. Similarly, the sCOD value observed in TSEP was found to be 20.75% higher than TSP and 9.35% higher than EP. The macromolecules such as proteins and carbohydrates released in the sludge during different pre-digestion methods are presented in Fig. 4b.

The soluble protein content for TSP, EP and TSEP measured were found respectively to be 667 ± 30, 743 ± 20 and 924 ± 20 mg/L, which are considerably higher than undigested sludge (492 ± 30 mg/L). Also, the solubilized carbohydrate content for TSP, EP and TSEP determined were found respectively to be 460 ± 20, 516 ± 20 and 667 ± 10 mg/L, which are significantly higher than undigested sludge (300 ± 20 mg/L). Hence, SS reduction, sCOD and



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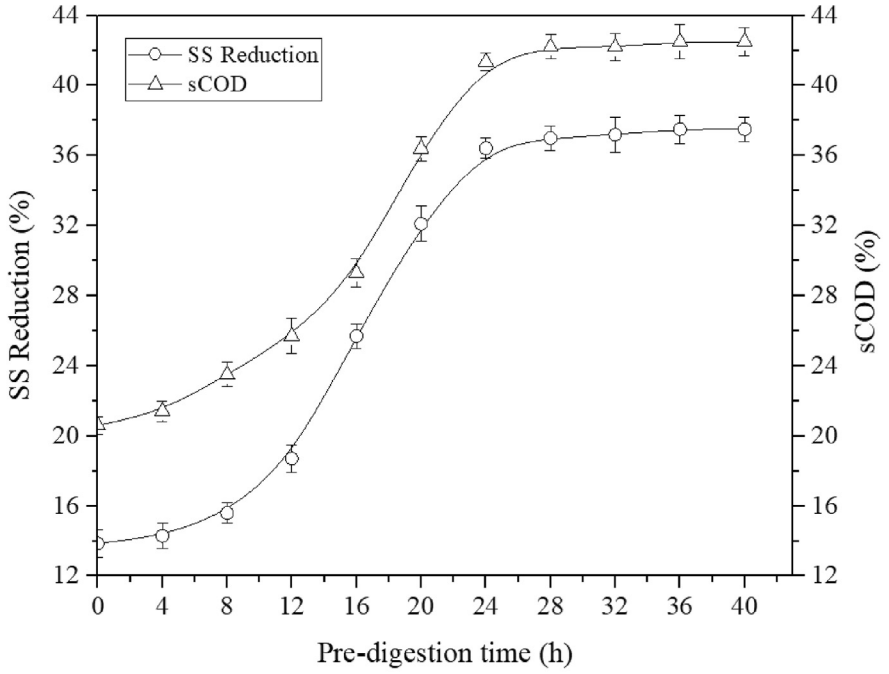

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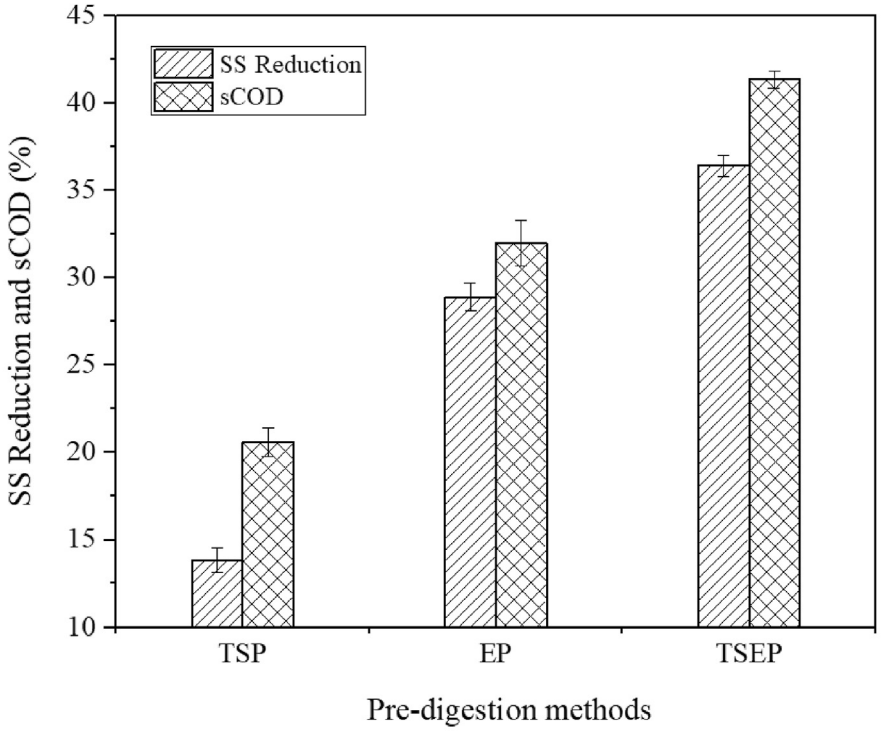
Fig. 3. a: Influence of thermo-sonic assisted enzymatic pre-digestion time on soluble proteins and carbohydrates release (using 20% MGE dosage, Temperature at 40 °C). **b:** Optimization of thermo-sonic assisted enzymatic pre-digestion time for MWAS hydrolysis (using 20% MGE dosage, Temperature at 40 °C).

organic fraction release efficiency were in the sequence of TSP < EP < TSEP. The better results exhibited by TSEP method may be due to the combined effect of thermo-sonic as well as the influence of multi-hydrolytic activities of the MGE.

3.4. Growth dynamics, biomass and lipid productivity

For scaling up of biodiesel production from a microbial resource, the propagation rate of microbes, biomass yield and appropriate lipids content are the vital parameters to be considered. Hence, the



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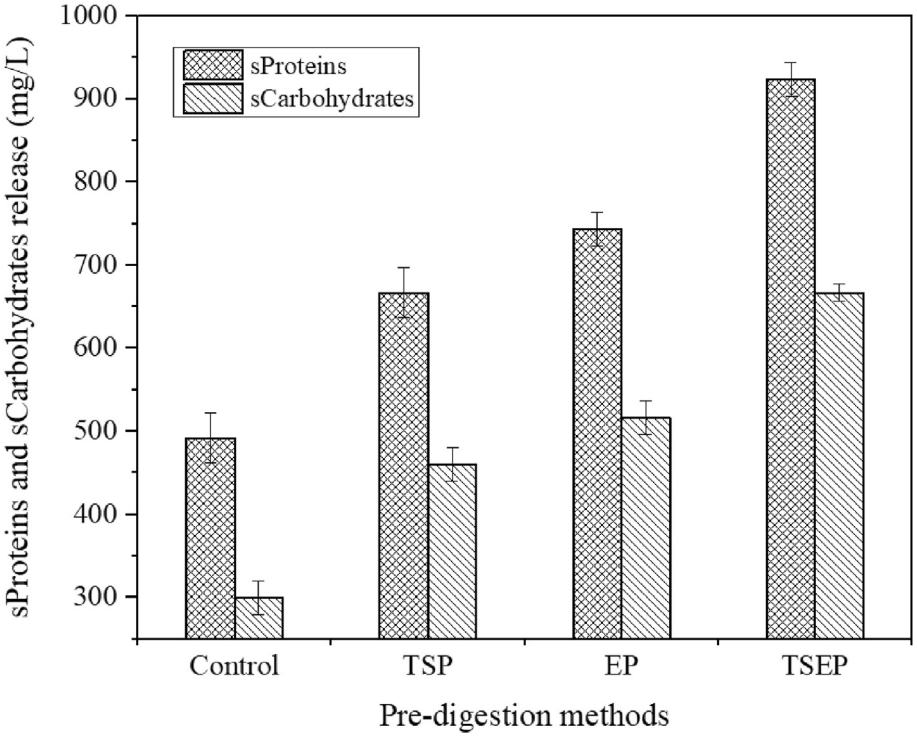

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Fig. 4. a: SS reduction and COD solubilization as a function of different pre-digestion methods (TSP, Thermo-sonic pre-digestion; EP, Enzymatic pre-digestion; TSEP, Thermo-sonic assisted enzymatic pre-digestion). **b:** Soluble proteins and carbohydrates release as a function of different pre-digestion methods.

biomass would be taken as a suitable feedstock for preparing biodiesel only when it associated with high lipid content. In this study, the oleaginous yeast *N. liquefaciens* NITTS2 was cultivated in the TSEP medium. The yeast growth rate, carbohydrate utilization, biomass yield and lipid content were measured at regular intervals for 168 h and the values achieved are presented in Fig. 5.

It can be seen from Fig. 5 that the proliferation of *N. liquefaciens* NITTS2 begins well after 24 h and then exponential growth phase is extended up to 144 h. The calculated specific growth rate was found to be 0.86 day^{-1}. The biomass, as well as lipid content, are logarithmically increased with culturing time up to 144 h and then nonet increment is observed during the stationary phase. The maximum achieved biomass yield and lipid content were found respectively to be 17.85 ± 0.64 g/L and 65.43 ± 1.60% in the TSEP medium at optimum culturing conditions. The biomass yield and lipid content in the TSEP medium measured were 3 and 3.18 times, respectively higher than un-predigested medium. This may be because of the high availability of dissolved nutrient fractions released from the MWAS during TSEP pre-digestion. TSEP enhanced the biomass yield of 4.27 g/L and lipid content is 16.03% higher than the culture control medium [27]. The biomass and volumetric lipid productivity obtained respectively were 2.98 ± 0.10 and 1.95 ± 0.005 $gL^{-1}day^{-1}$. These results reveal that the lipid production in TSEP is 10.26 and 1.74 times correspondingly higher than un-digested and culture control medium. This may be due to the availability of solubilized nutrient content contributes to the growth as well as the lipid build-up of the *N. liquefaciens* NITTS2 in the TSEP medium [32].

3.5. Consumption of nutrients by yeast strain for lipid production

In order to understand the nature of supplement take-up by *N. liquefaciens* NITTS2 in TSEP medium for lipid production, the nutrient fractions present in the medium were analyzed before and after batch cultivation and presented in Table 1.

The MWAS mainly consisted of macromolecules namely proteins and carbohydrates because these molecules typically composed of microbes which include fungi, bacteria and protista [44]. The consumption of protein and carbohydrate by *N. liquefaciens* NITTS2 respectively were nearly 91.33 and 91.62%. This reveals that the TSEP medium was efficiently used by the yeast strain, which may be because the microbe was screened and isolated from the same environmental conditions. The consumption rate of carbohydrate and protein by *N. liquefaciens* NITTS2 were found respectively to be 129.16 ± 30 and 8.05 ± 0.10 mg/h at optimum culture conditions of pH 6.3, agitation speed 160 rpm and temperature 30 °C.

The carbohydrate was considered to be a chief substrate in *de novo* lipid metabolism of microorganisms to synthesis the triglycerides as an end product [45]. It is generally recorded that the oleaginous yeast species primarily utilize the carbon sources for their proliferation and lipid production along with inadequate other inhibitory supplements, for example, nitrogen in their cultivation medium [12,18]. During the pre-digestion, the DNA

Table 1
The nutritional composition in TSEP medium before and after batch cultivation of *N. liquefaciens* NITTS2.

Chemical composition	Before cultivation	After cultivation
Carbohydrate (mg/L)	20300 ± 500	1700 ± 200
Protein (mg/L)	1270 ± 50	110 ± 20
Nitrogen (mg/L)	630 ± 40	1.2 ± 0.4
Carbon (mg/L)	18500 ± 1300	800 ± 50
DNA (mg/L)	240 ± 30	0.7 ± 0.2
Calcium (mg/L)	10.2 ± 1.5	0.8 ± 0.1
Sodium (mg/L)	14.5 ± 1.7	0.8 ± 02
Phosphorous (mg/L)	30.7 ± 1.8	1.5 ± 0.5
Potassium (mg/L)	19.3 ± 0.6	1.0 ± 0.4
Chloride (mg/L)	8.5 ± 1.5	0.6 ± 0.2
Iron (mg/L)	2.3 ± 0.5	0.6 ± 0.2
Sulphur (mg/L)	7.2 ± 0.4	1.2 ± 0.3

All values presented are the mean ± standard error of three measurements (n = 3). The batch cultivation of *N. liquefaciens* NITTS2 was carried out for 144 h in TSEP medium for lipid production. The maintained optimum culture conditions were pH 6.3; temperature 30 °C; agitation speed 160 rpm. The obtained biomass yield and lipid content were 17.85 ± 0.64 g/L and 65.43 ± 1.60%.

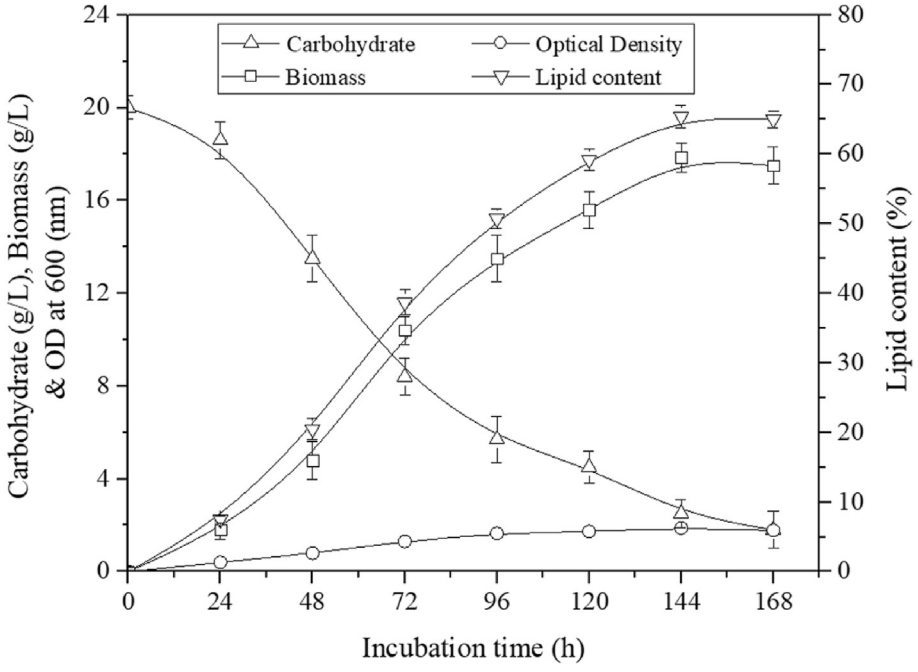

Fig. 5. Profile of biomass production and respective lipid content, carbon source consumption, and optical density of *N. liquefaciens* NITTS2 cultivated in TSEP medium.

molecules were released in the liquid phase from the microbial source existing in the sludge. The achieved amount of initial concentration in the production medium was 240 ± 30 mg/L and remained in the medium after cells harvesting was 0.7 ± 0.2 mg/L. This makes the sense that the isolate has the ability to utilize the pentose sugars effectively. The same genus of yeast such as *C. albidus* and *C. curvatus* were similarly found to utilize pentose sugars present in the basal medium and acid hydrolysate of sweet sorghum bagasse respectively, which were used as culturing medium for lipid synthesis [46,47]. It can be seen from Table 1 that a significant amount of micronutrients exist in the TSEP medium were consumed by *N. liquefaciens* NITTS2 for their metabolism. Table 2 presents the comparison of the lipid yield for different yeast species along with the various substrates and growth conditions.

It can be observed that the strain *N. liquefaciens* NITTS2 cultivated in the TSEP medium has yielded high lipid due to adequate availability of nutrients. Also, it can be noticed that *N. liquefaciens* NITTS2 has high lipid content when cultivating in this low-cost substrate, which is available in large quantities around the world. Thus, the chemical composition of TSEP medium can be considered as an effective nutritional source for microbial cultivation for lipid synthesis.

3.6. Ultrasonic-assisted lipid extraction

To improve the lipid extraction efficiency using solvents, pretreating methods namely homogenization, bead milling, ultrasonication or microwave heating can be applied for cell disruption and also these lead to reduce the solvent volume required for extraction practices [48]. The yeast biomass with extracting solvent was exposed to ultrasonication at different temperature (20, 30, and 40 °C) for different time intervals. The influence of different temperature on the recovery of lipid as a function of ultrasonication time is depicted in Fig. 6.

It is noticed from this figure that lipid yield increases exponentially at each temperature until 20 min of sonication time and then the negligible improvement is noticed up to 30 min. The maximum lipid yield recovered were 52.0 ± 2.2, 65.4 ± 1.4 and 65.8 ± 1.6% respectively for 20, 30, and 40 °C. A similar observation was recorded by Zhang et al. [49] where the lipids were extracted from *Trichosporon oleaginosus* biomass. At 20 °C the lipid yield was less and similar results were acquired for 30 and 40 °C. The observed maximum lipid recovery in control (not exposed to ultrasonic pre-treatment) was 7.2 ± 1.4% till 30 min at ambient temperature. The lipid extracted with the aid of ultrasonication was found to be 9.08 fold higher than the control experiment. The shear force produced during ultrasonication is accountable for rupturing the cell wall, and the ultrasonication assisted lipid extraction considerably reduced process time. Whereas, the conventional method requires more than 12 h to achieve the same outcomes [49]. Thus, 20 min ultrasonication time at 30 °C was considered as optimum for effective lipid extraction from yeast biomass.

3.7. Characterization of lipid and FAMEs

The profile of extracted lipid was analyzed by GC-MS and the corresponding chromatogram is displayed in Fig. A1 (see supplementary data). It is revealed that the lipid of *N. liquefaciens* NITTS2 primarily consisted of oleic acid (37.2%), palmitic acid (24.4%), linoleic acid (23.2%), stearic acid (12.5%), linolenic acid (1.4%) and myristic acid (0.6%). This lipid profile was quite similar to jatropha oil and also notably comparable to the lipids of *C. vishniaccii* of the same genus, which was cultured in the hydrolysate of paper mill sludge after sonication as a cultivation medium [50]. According to the determined fatty acids profile, the lipid synthesized by *N. liquefaciens* NITTS2 using TSEP medium could appropriately be used for the biodiesel production. FAMEs was prepared from the lipids using methanol with an enzyme catalyst which yielded 88.45 ± 1.2%. NMR is a multipurpose spectroscopic technique mostly used to find the structure of chemical compounds, and the ^{1}H NMR spectroscopy is extensively used for the characterization of FAMEs formed [51]. The obtained FAMEs was examined by ^{1}H NMR spectroscopy, and the result is illustrated in Fig. A2 (see supplementary data). A typical strong singlet peak at 3.610 ppm observed is concerned to methoxy protons, and at 2.251 ppm detected peak was corresponding to α-methylene protons (α-CH_2) of methyl esters. These two characteristic peaks perceived in the ^{1}H NMR spectrum are the credentials for the corroboration of FAMEs present in the sample. The terminal methyl protons and methylenes of the carbon chain have exhibited a peak at 0.952 and 1.210 ppm, respectively. The multiplet at 1.554–1.622 ppm is related to b-carbonyl methylenes and the triplet at 2.250–2.298 ppm is

Table 2
Different economically viable feedstock for various yeast species cultivation to lipid production.

Cultivation medium	Microbes involved	Optimum process parameters	Lipid yield (g/L); Lipid content (%w/w)	Reference
Rice straw hydrolysate	*Trichosporon fermentas*	pH 6, 25 °C, 160 rpm	11.5; 40.1	[53]
Molasses	*Candida lipolytica*	pH 5, 30 °C, 100 rpm,	NA; 59.9	[54]
Raw glycerol	Candida oleophila ATCC 20177	pH 6.1, 28 °C, 185 rpm,	1.44; 15.3	[55]
Corncob hydrolysate	*Trichosporon coremiformii*	pH 4.5, 28 °C, 150 rpm	7.7; 37.8	[56]
Waste sweet potato wine hydrolysate	*Trichosporon fermentas*	pH 4.5, 28 °C, 150 rpm	9.6; 35.6	[57]
Sugar beet molasses	*Rhodotorula glutinis* TR29	pH 5, 25 °C, 200 rpm	10.5; 64.8	[58]
Paper mill sludge	*Cryptococcus vishniaccii* MTCC 232	pH 7, 25 °C, 200 rpm	7.8; 53.4	[50]
Groundnut shell hydrolysate	*Cryptococcus psychrotolerans* IITRFD	pH 7, 25 °C, 200 rpm	6.33; 46.0	[59]
Thermo-chemo-sono pre-digested MWAS	*lipomyces starkeyi* MTCC 1400	pH 5.5, 32 °C, 150 rpm	11.3; 64.3	[18]
TSEP MWAS	*Naganishia liquefaciens* NITTS2	pH 6.3, 30 °C, 160 rpm	11.68; 65.4	This study

NA: Data Not Available; All cultivations of yeast species in the different culture medium for lipid production cited in the table were performed in batch mode with different time durations.

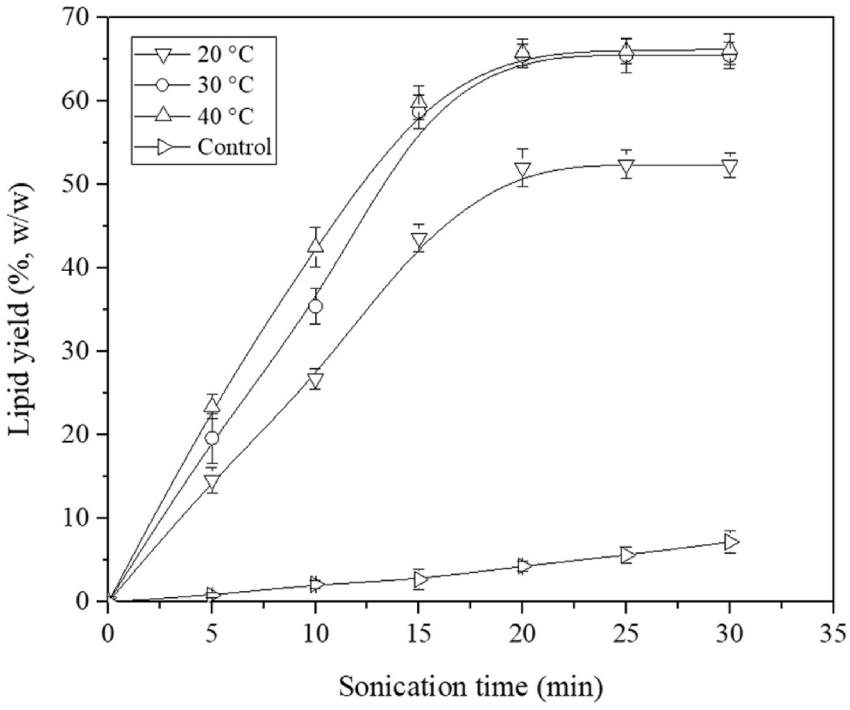

Fig. 6. Effect of ultrasonication time on lipid extraction from *N. liquefaciens* NITTS2 biomass at different temperature (20, 30 and 40 °C).

assigned to a-carbonyl methylenes. These determined results are comparable to previously described ^{1}H NMR spectrum confirmations of the FAMEs produced from different oil sources [32,52]. The purity of biodiesel produced from yeast lipids calculated using the integration value of the methoxy protons and α-methylene protons of the methyl esters measured from Fig. A2 (see supplementary data) was 89.90%. Hence, good quality biodiesel was accomplished by enzymatic transesterification of extracted lipids from a novel oleaginous yeast strain cultivated in a sustainable medium.

4. Conclusions

Thermo-sonic assisted enzymatic pre-digestion enhanced the solubilization of sludge. The maximum SS reduction and sCOD were observed respectively to be 36.42 ± 0.7 and 41.35 ± 0.5% at optimum conditions. Soluble proteins and carbohydrates increased during combined sludge pre-digestion were 432 ± 20 and 367 ± 10 mg/L respectively higher than the undigested sludge (492 ± 30 and 300 ± 20 mg/L, respectively). The concentration of suspended solids reduction, soluble COD and organic fraction release efficiency perceived were in the sequence of TSP < EP < TSEP. The TSEP medium supplemented adequate nutrients for the *N. liquefaciens* NITTS2 to accumulate a greater quantity of lipids (65.43 ± 1.60%) at pH 6.3, agitation speed 160 rpm and temperature 30 °C. The lipid extracted with the assistance of ultrasonication was found to be 9.08 times higher than the control experiment. The extracted lipids have excellent burning value because of oleic acid (37.2%), palmitic acid (24.4%) and linoleic acid (23.2%). Thus, the combined pre-digestion made the MWAS as a potential substrate for microbial lipid synthesis for biodiesel production and other industrial applications.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2019.03.040.

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